UC Riverside

UCR Honors Capstones 2022-2023

Title

Fluorescent Tracking Of Horizontal Gene Transfer In Gut Microbes

Permalink

https://escholarship.org/uc/item/06j5j6tj

Author

Hofstedt, William

Publication Date

2023-06-16

eScholarship.org Powered by the California Digital Library
University of California

FLUORESCENT TRACKING OF HORIZONTAL GENE TRANSFER IN GUT MICROBES

By

William Grimberg Hofstedt

A capstone project submitted for Graduation with University Honors

May 05, 2023

University Honors

University of California, Riverside

APPROVED

Dr. Patrick Degnan

Microbiology and Plant Pathology

Dr. Richard Cardullo, Howard H Hays Jr. Chair

University Honors

ABSTRACT

Fluorescent Tracking of Horizontal Gene Transfer in Gut Microbes

Abstract: The human gut microbiome is diverse both in composition and in function. Further, changes in the microbiome can contribute to both health and disease. Many of these microbes can swap genes in a process called horizontal gene transfer (HGT). This allows microbes to gain advantageous phenotypes such as antibiotic resistance, the ability to consume different nutrients, and/or produce cell coatings that allow them to avoid a host's immune system. Tracking these transfer events is challenging, particularly if it is unclear which parts of the genome of a microbe can or can't be transferred. Here, I propose examining transfer events using anaerobic fluorescent proteins (AFPs) combined with transposon mutagenesis. First, I tested a panel of 4 novel AFPs for the ability to be expressed and detected in the model gut microbe *Bacteroides thetaiotaomicron* VPI-5482. I then used transposon mutagenesis to randomly insert the AFPs into several species of gut microbe (donors) and after co-culturing them with unmodified species (recipients), in an attempt to identify recipient cells that acquired the AFPs. Once confirmed, regions of mobile DNA responsible for transferring the AFP can be characterized. This approach offers a novel way to rapidly detect HGT events in complex gut microbe communities and find new functional genes that contribute to a microbe's ability to colonize the human gut.

ACKNOWLEDGMENTS

I would like to extend my deepest gratitude to my faculty mentor Dr. Degnan and my graduate student mentor Jericho Ortañez for their guidance and support. Much of the work leading up to this project is based on unpublished research from members of the Degnan lab. I would also be remiss in not mentioning the other graduate students in the Degnan lab who have given me advice to this point, including David Crago and Disha Dutta.

TABLE OF CONTENTS

INTRODUCTION:

The bacteria within the microbiome of the human gut are critical for human health (Bull & Plummer, 2014). Many of these microbes are members of the Gram-negative phylum Bacteroidota. The ability of these microbes to survive and colonize in the human gut (e.g., their fitness) can be influenced by mobile genetic elements (MGEs). These genetic elements can give microbes new functions or abilities that in turn can alter the health of their human hosts. This project focused on identifying functional MGEs of *Bacteroides thetaiotaomicron* VPI-5482. By identifying the locations of these active genes, it becomes more efficient to study their potential structural, metabolic, or regulatory functions.

Genes encoding Vitamin B_{12} transporters are one example of genes tied closely to MGEs. Vitamin B_{12} transporters are critical for the growth of most gut Bacteroidota with some microbes encoding up to 4 sequence diverse copies of these transporters (Degnan et al., 2014, Frye et al., 2021). These diverse transporters function in different conditions and interact with different structural variants of vitamin B_{12}. Homology-based analyses identified a handful of putative MGEs that were partly responsible for the microbes encoding their diverse B_{12} transporters (Degnan et al., 2014). These putative MGEs were tested, and some were confirmed to be able to move between microbes in the gut environment (Frye et al. 2021).

Genome sequencing and homology-based analyses can identify putative MGEs, however demonstrating that they are transmissible requires genetic experiments. Furthermore, these experiments require selectable marker systems. These are genes inserted into DNA sequences of interest to cause some kind of phenotypic change in the host organism. The resulting change in phenotype (e.g., color, fluorescence, growth) indicates whether the gene sequence of interest is present in the host and being expressed. Traditionally, selectable markers in microbes have been

antibiotic-resistance genes. The use of antibiotic resistance allows for the easy selection of microbial cells that have successfully taken on the intended genetic material (because those that do not will die). However, this methodology has its limitations. Many bacterial species of interest have a wide variety of antibiotic-resistance genes, an evolutionary result likely affected by the misuse of antibiotics against pathogenic strains (Wang et al., 2000).

The issue of antibiotic resistance can be avoided by using fluorescent selectable markers instead. While tools such as green fluorescent protein (GFP) have been effective for many purposes, they are limited to aerobic environments (those with oxygen). In recent years, a new methodology focused on two bilin-binding fluorescent proteins, UnaG and IFP2.0, was developed (Chia et al, 2020). These proteins can fluoresce in anaerobic environments, allowing for their use in studying gut microbes such as *Bacteroides thetaiotaomicron*. Flow sorting has also enabled scientists to check the fluorescence of individual cells by diluting suspended cell cultures significantly and then passing droplets the cultures past a laser at an excitation wavelength and, measuring the fluorescence of each drop, then sorting the drops in real time based on their fluorescent properties (BD Sciences, 2023).

This project focused on identifying mobile genetic elements within the Bacteroidota phylum. Most methods of doing so previously used targeted strategies as noted in the B_{12} example above. However, such methods were limited to confirming mobile genetic elements one at a time. Further difficulties arise due to the drastically varying transfer efficiencies of many MGEs, making it difficult to find less commonly transferred MGEs. An alternative to these older methods revolves around transposon mutagenesis.

Transposon mutagenesis involves the introduction of selectable marker genes to random places in the genome of the species of interest (Goodman et al, 2009). These donor populations

are crossed with a recipient species. In the donor population, enough random locations in the genome are marked that when any mobile genetic elements move from the donor species to the recipient species, at least one copy of the MGE that moved was marked with the selectable marker gene. This should result in the required change of phenotype to identify a recipient cell as having received an MGE from the donor population. The location of the selectable marker sequence in a recipient genome serves as a starting point for PCR amplification of the surrounding DNA. This surrounding DNA can then be sequenced and compared to known DNA sequences for the donor wild-type species to determine how much of the DNA in the recipient came from the donor. The DNA that moved represents a putative MGE. The goal of this project was to use transposon mutagenesis to randomly mark MGEs with fluorescent selectable markers and observe their mobilization and transfer among strains.

MATERIALS AND METHODS:

Cell Culture:

Bacteroides strains were grown in three conditions: Tryptone-yeast extract-glucose broth (TYG) liquid medium, liquid minimal medium (MM) (Martens et al., 2008), and 10% defibrinated horse blood supplemented Brain Heart Infusion (BHI-HB) agar plates. *Escherichia coli* S-17 cells were grown in liquid and agar LB medium. All liquid cultures, regardless of species, were prepared by adding a single isolated colony to five mL of media. Agar plates were prepared by pouring ~25-35 mL of BHI-HB into each of 100 x 15 mm round Petri dishes. When applicable, the following final antibiotic concentrations were used: tetracycline 2 μg/mL, gentamycin 200 μg/mL, and erythromycin 25 μg/mL.

Anaerobic growth took place in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). The injected anaerobic gas composition was 73% N_2, 20% CO_2, and 7% H_2. All cell cultures were grown at 37ºC aerobically or anaerobically when needed.

Conjugation Procedure:

Donor and recipient strains were streaked on BHI-HB plates to isolate individual colonies. Colonies were picked after 1-2 days of growth and then inoculated and grown in 5 mL of TYG for 16-20 hours. Recipient subcultures were prepared in 5 mL of media and diluted at a rate of 1:200, 1:500, and 1:750. 5 mL Donor subcultures were prepared in 5mL and diluted at a rate of 1:100 and 1:200. *E. coli* strains were prepared aerobically during these steps while all *Bacteroides* strains were prepared anaerobically.

A dilution of the donor and a dilution of the recipient that were both within an optical density at 600 nm (OD_{600}) of 0.1 of each other while still within the 0.3 to 0.6 total OD_{600} range

after at least 3-4 doublings were set aside to be crossed. 5 mL of these donor and recipient cultures were spun down into pellets using 15 mL falcon tubes, at 4,000 rpm for five minutes (2,061 x g).

The supernatants were removed, and the pellets were each washed with 1 mL TYG. The cells were spun down into pellets again under the same conditions to be washed twice more.

After the last washing, the pellet of the recipient was resuspended in 1 mL of fresh TYG. That same 1 mL was then used to resuspend the donor cell pellet. The combined, resuspended pellets were then deposited onto a BHI + 10% horse blood agar plate. The plate was repeatedly tilted to evenly distribute the fluid.

The plate was incubated agar side down and in a 37ºC incubator for 16-24 hrs. This was typically done in an anaerobic incubator except when crossing *B. thetaiotaomicron* and *E. coli*. This exceptional case allowed an *E. coli* lawn to form which protects the *B. thetaiotaomicron* beneath it from the surrounding oxygen, allowing it to grow.

Most of the bacterial biomass from the plate was resuspended in 5 mL of TYG. Serial dilutions of this resuspended material were plated for two days, and then colonies were collected. Each dilution was plated onto BHI-Blood Agar which contained antibiotics in order to kill donor cells and non-transconjugant recipients.

Overnights of the transconjugants were grown in TYG and antibiotics. Frozen permanents were made with a 20% final concentration of glycerol and stocked at -80ºC.

Generating Fluorescent Transconjugants:

The efficacies of four potential fluorescent selectable markers generated by the Degnan lab were tested in *B. thetaiotaomicron*: pNBU2_ErmG_UnaG, pNBU2_ErmG_iLov, pNBU2_ErmG_BtCreiLov, and pNBU2_ErmG_EcFbFP. UnaG fluoresces green when bound to

bilirubin making it a bilin-binding fluorescent protein (BBFP)(Kumagai et al, 2013). iLov, BtCreiLov, and EcFbFP are all Flavin-based Fluorescent Proteins (FbFPs). FbFPs are characterized by their Flavin precursors and cyan-green anaerobic fluorescence (Mukherjee et al., 2014).

Prior to this project, each plasmid was ligated, transformed, and sequence confirmed into *E. coli* by the Degnan Lab. During this procedure, each plasmid was transferred into *B. thetaiotaomicron* strains through bacterial conjugation. In *E. coli*, pNBU2 plasmids are self-replicating, while in *B. thetaiotaomicron* strains, pNBU2 plasmids are "suicide" plasmids that integrate into the genome (Martens et al. 2008). It was known from previous experiments that there are two possible attachment sites in the *B. thetaiotaomicron* genome for the pNBU2 plasmid (Wang et al., 2000). Therefore, transconjugants were selected for using antibiotic resistance were then confirmed with polymerase chain reactions (PCR as described later in this work) designed to amplify the unmodified attachment sites, identifying the modified attachment site (with the integrated plasmid) through a lack of amplification. Specific attention was paid to the transconjugants producing the fluorescent protein denoted as UnaG to determine the attachment site of the fluorescent protein gene cassette within *B. thetaiotaomicron.* This was necessary to determine if the attachment location within the cell genome influenced the intensity of fluorescence.

To screen the strains by PCR, first DNA was extracted via phenol-chloroform extraction that was a variation of that performed in Campbell et al (2020). Each culture was pelleted (2061g x 5') and resuspended in 1-1.5 mL Tris-EDTA (TE). 1.65 µL 20% sodium dodecyl sulfate (SDS), 7.5 µL 20 mg/mL Proteinase K, and 500 µL of TE were added to each resuspended pellet. Each test sample was incubated at 50 ºC for 2 hrs. Phase lock tubes were prepared by

centrifugation at 12,000 g for 30 sec. A volume of Phenol: Chloroform equal to the volume of each particular sample was added to each sample tube. Samples were spun in a microfuge at 12,000 g for 5' to separate the phases. The aqueous phase was transferred to a new 1.5 mL tube, and 11.1 µL of 3M NaOAc per 100 µL of DNA solution was added to each sample. Each sample was vortexed to mix and was added 2 times their volumes in 100% EtOH and incubated at -20ºC for 9 days. Samples were then centrifuged at 15,000 g for 30' at 4ºC. The pellets were washed with 500 µL of 70% EtOH. After the final wash, the samples were pelleted, and the pellets were dried by inverting the tubes for ten minutes. Samples were resuspended in 200 µL of TE and stored at 4°C.

Then PCR for each of the two attachment sites (*att1* and *att2*) was performed by combining the following reagents into one PCR tube for each of the samples being amplified: 1 µL of buffer, 0.2 µL of dNTPs, 0.4 µL Forward Primer, 0.4 µL Reverse Primer, 0.05 µL taq Polymerase, 6.95 µL Water. One tube of each PCR procedure set had the wild-type genetic material of *B. thetaiotaomicron* added. These samples served as positive controls (as both attachment sites were amplified). Another tube of each set had no DNA added to serve as negative controls (as nothing was amplified). DNA was spun down for 5 seconds using a mini centrifuge. Tubes were placed in the thermocycler. The cycling conditions were as follows: 95ºC for two min. 95ºC for 30 seconds. 49.5ºC for 30 seconds. 72ºC. 1 minute. Return to the 49.5ºC step, 30 times. 72ºC for 5 min. 4ºC, until removal from the device ~30 minutes later. Amplified DNA was moved to the -20ºC freezer.

Then, gel electrophoresis was performed using 1% agarose gels in 1X TAE running buffer. Controls, samples and molecular weight standard ladders were pre-stained with GelRed

and mixed with Orange G + 60% glycerol loading dye and run at 120V for 30 minutes. Images were taken and stored using a BioRad imaging station.

Finally, after attachment site determination based on the PCR testing, multiple colonies that had an insertion at attachment site one and multiple colonies that had an insertion at attachment site two were tested for fluorescence.

Fluorescent Detection:

Potential fluorescent transconjugants were cultured in liquid media and analyzed for fluorescence on a ClarioStar plate reader (BMG). Initial fluorescence testing occurred at the following excitation/emission wavelengths for each fluorescent selectable marker: UnaG was 498-8/527-8 with a gain of 2500, and all three of the FbFPs (EcFbFP, iLov, and CreiLov) were all 450-15/500-20 with a gain of 250. The experiment was continued using only one of these four: UnaG. Later, a fifth fluorescent protein, IFP, was also used with fluorescence being detected at 675-15/720-20 with a gain of 3000.

Overnight cultures of the required strains were grown as described above, then washed in minimal medium, normalized based on their OD_{600} values, and diluted into fresh media in 96-well microtiter plates. Bilirubin and biliverdin, the activating ligands of the UnaG and IFP proteins respectively, were supplied during the UnaG and IFP testing using concentration ranges from 0.024-2.4 μM. Testing was performed with, and without cells, both groups had subgroups with and without bilirubin/biliverdin to ensure it was not presenting a confounding factor. All test groups were prepared in triplicate and averaged for results.

tRNA Adjustment of UnaG:

Expression of proteins in foreign (heterologous) hosts can be affected by the identity of tRNA codons in the mRNA that code for the protein. The laboratory generated two UnaG

expression vectors that were each independently "codon adapted" in the hopes of increasing their effectiveness as selectable markers in *B. thetaiotaomicron*. Three groups of *B. thetaiotaomicron* cells were used to test the efficacy of the codon adaptation. The first group was simply a negative control with no added genetic material, while the second and third groups were conjugated with the original (Chia et al. 2020) and Degnan-Lab versions of the tRNA-adjusted gene sequence for UnaG, respectively. These transconjugants were cultured in liquid media and analyzed for fluorescence as described above, and fluorescence was compared between the two UnaG versions.

Transposon Mutagenesis of The Donor:

The Degnan laboratory inserted the UnaG gene and its associated promoter into the pSAM transposon cassette (Goodman et al, 2009). *E. coli* was used to introduce this UnaG transposon cassette into random locations in the *B. thetaiotaomicron* genome through conjugation. Most transconjugants are expected to have one inserted cassette, and the location should vary widely from cell to cell. A 'donor' population of individually mutagenized *B. thetaiotaomicron* cells was selected using Erm^R.

Preparation of the Recipient Strains:

Potential recipient strains were created by conjugating the pNBU2 integrating plasmid encoding the genes for Tetracycline resistance (Tet^R) and IFP with *B. fragilis* and *B. ovatus* as described above. Confirmed transconjugants were stocked as frozen permanents as described above.

Crossing of the Donor and Recipient Strains:

The donor population (Transposon Mutant Library (TnMn) *B. thetaiotaomicron* population with UnaG) and the recipient population (B. *ovatus* with IFP) were crossed twice.

Conjugations were performed with thawed *B. thetaiotaomicron* TnUnaG pool as donors and IFP labeled recipients as described above. CFUs of donors were calculated in order to estimate conjugation efficiencies. Between each attempt, multiple conditions were changed including different colonies from the original creation of the recipient strain were used and the co-plating time was increased from 20 hours to 28 hours. Due to unexpected difficulties at this stage, the use of the flow sorter was never initiated.

RESULTS:

TYG medium is a rich growth medium that supports the growth of many Bacteroidota. However, when measuring fluorescence, TYG possesses notable background fluorescence, which is why minimal media was used during the majority of fluorescence tests (Figure 1). Often the background fluorescent signal decreases over time, suggesting part of the signal is metabolized by the microbes during growth. Despite indications that FbFPs can fluoresce anaerobically and the use of a very strong promoter sequence, no significant fluorescence was detected during the course of this project from EcFbFP, iLov, or CreiLov (Figure 1). The only successfully fluorescing proteins in *B. thetaiotaomicron* were UnaG and IFP.

In a head-to-head comparison of the Degnan Lab codon adapted form of UnaG was shown to be more effective in *B. thetaiotaomicron* than the previously published version of the protein (Figure 2).

Furthermore, the data collected in this procedure suggests that fluorescence was not correlated to attachment location despite two clusters of intensities forming between all parallel executions of this procedure occurring. It is unclear what experimental variables may contribute to the ~2-fold difference in fluorescence among strains (Figure 3).

The donor population (Transposon Mutant Library (TnMn) *B. thetaiotaomicron* population with UnaG) and the recipient population (*B. ovatus* with IFP) were conjugated two times. However, neither mating resulted in colonies possessing the expected antibiotic resistance (Erm^R+Tet^R; Figure 5). Further attempts to conjugate the pNBU2_ermG_IFP plasmid into *B. fragilis* were also unsuccessful.

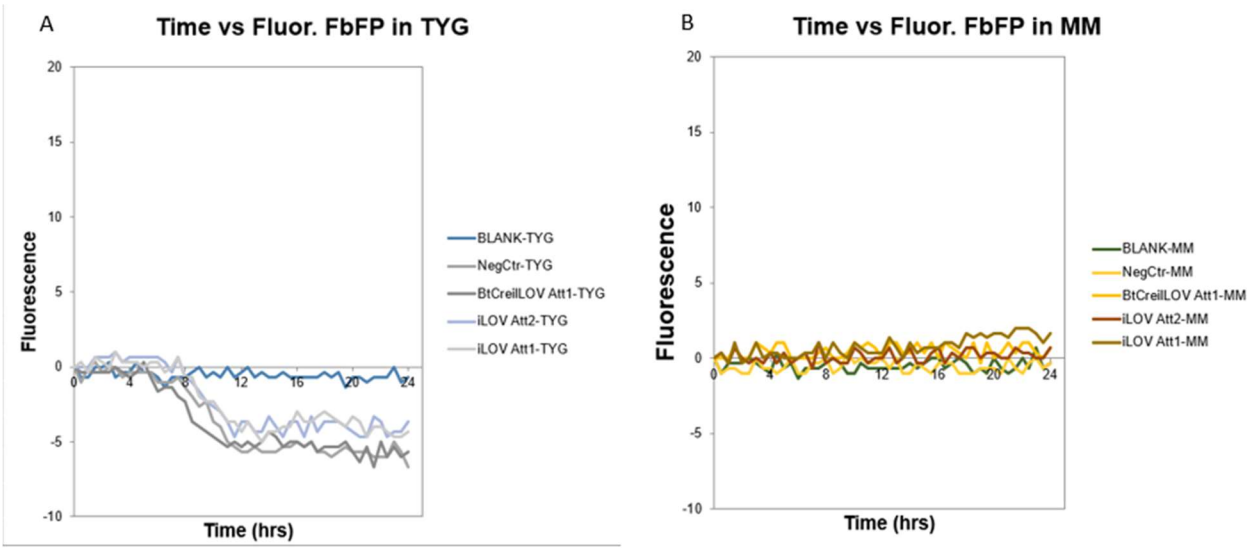

Figure 1: **Testing Anaerobic Fluorescent Proteins**. Potential anaerobic fluorescent proteins iLOV and BtCreiiLOV were integrated into *B. thetaiotaomicron.* Fluorescence was monitored during 24 hrs of growth in (A) TYG medium and (B) minimal medium. Two negative controls were included: *B. thetaiotaomicron* strain without a fluorescent gene and a Blank media only (e.g., no cells). Three technical replicates test samples were averaged to create each data curve.

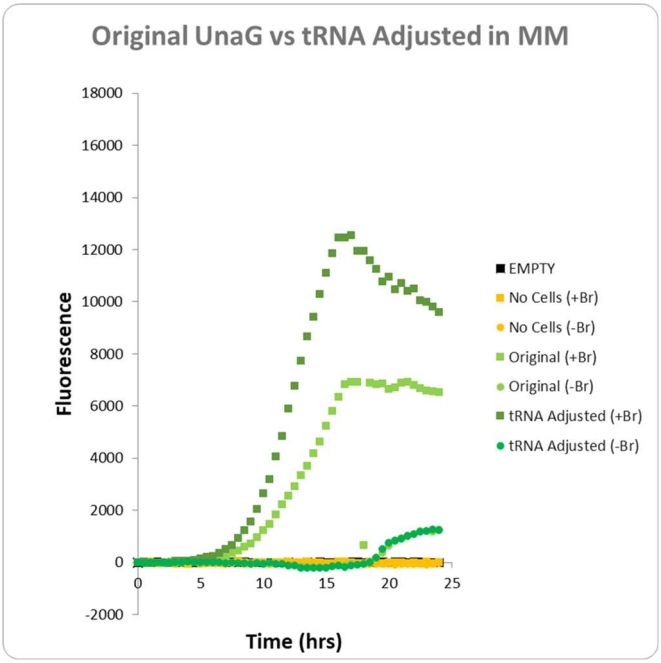

Figure 2: **Fluorescence of UnaG and newly tRNA Adjusted UnaG**. The original UnaG was technically already codon adjusted (Chia et al. 2020), but the Degnan Lab performed another tRNA adjustment on UnaG. These are referred to as 'original' and 'tRNA Adjusted' respectively for simplicity. These are both tested for fluorescence over time, normalized by OD_{600}. Each curve is the average of three technical replicates.

Table 1: **Summary of PCR attachments site screening of strains expressing UnaG.**

	First PCR Test			Second PCR Test		
Sample	**Att1 PCR**	**Att2 PCR**	**designation**	**Att1 PCR**	**Att2 PCR**	**designation**
#0	+	+	?	-	-	?
#1	Not Tested	Not Tested	Not Tested		+	1
#2			?	+	-	**2**
#3	+	+	?	-	+	1
#4	+		2	+	-	**2**
#5	-	-	?	-	+	1
#6			?	+	-	**2**
#7	+		2	+	-	**2**
#8			?	-	+	1
#9			?	-	-	?
#10	+		2	+	-	**2**

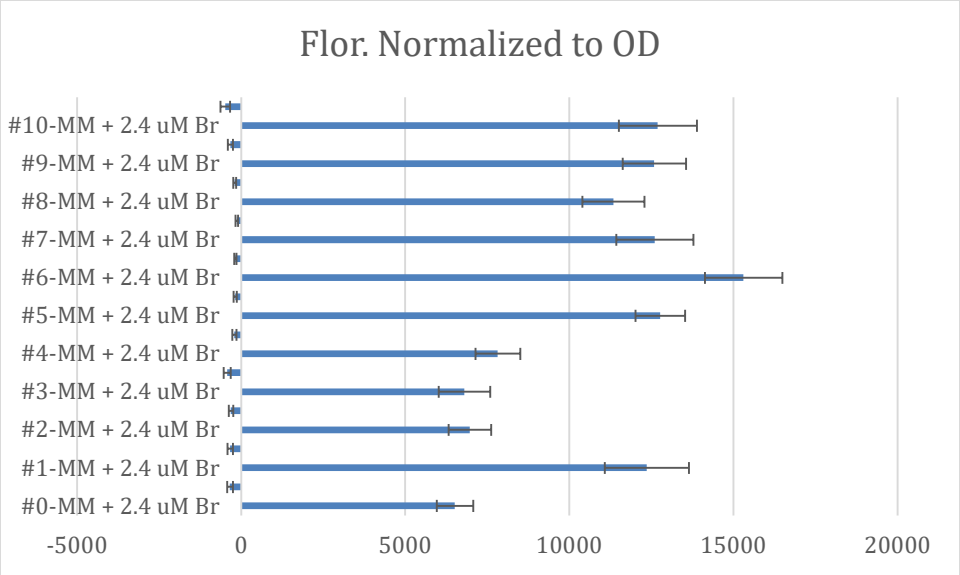

Figure 3: **Fluorescence of UnaG Tagged Strains**. Ten transconjugant strains were tested for fluorescence over time, normalized by OD_{600}. Strains correspond to those in Table 1. Each bar is the average of three technical replicates and the error bars represent the standard deviation.

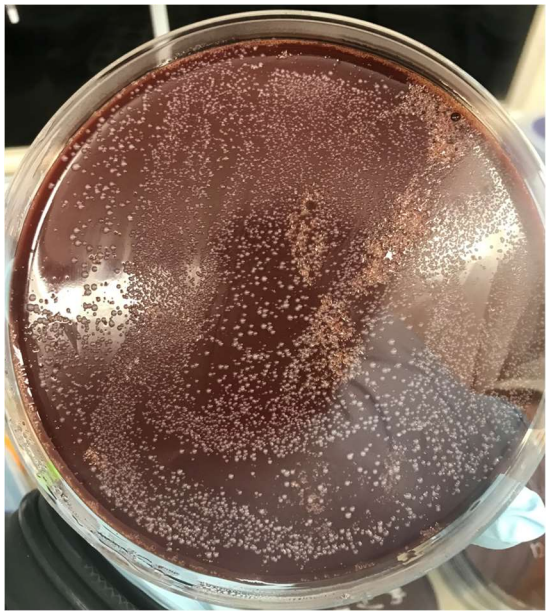

Figure 4: **Donor Colonies**. Seven plates, all identical to the one shown, were harvested. Thousands of successful transconjugant colonies were selected for using the antibiotic resistance paired with the fluorescent protein UnaG. These colonies were mixed and stored to form the transposon mutant library (TnMn) that would later form the donor population for the final crossing.

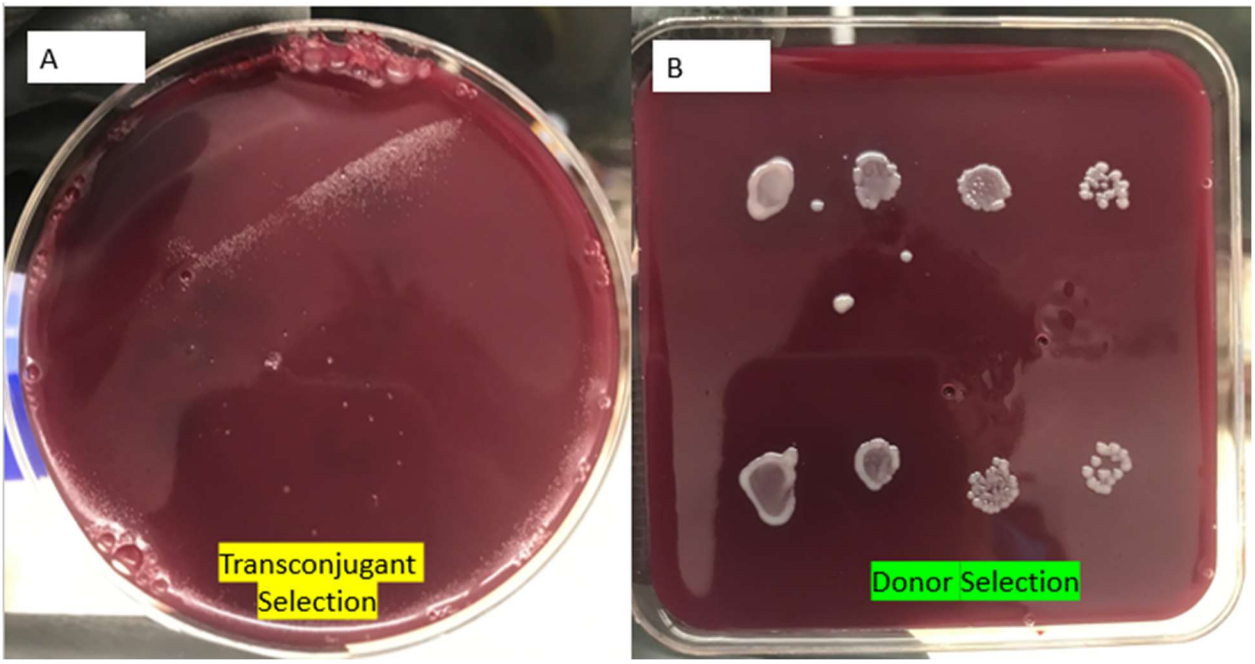

Figure 5: **Gene Transfer Attempt #1**. Four total duplicates of A had antibiotics, only allowing transformed recipient cells to grow, none of which did. B is a ten-fold serial spot dilution of the donor cells ending in a 10^{-8} factor dilution in the lower right. The second attempt was similar in results.

DISCUSSION:

Fluorescent Reporter Selection:

Five potential fluorescent selectable markers were studied in this work. Previous works suggested that all five of these potential fluorescent selectable markers were functional. However, three were not observed as functional in this work for unknown reasons. Previous research conducted by Degnan Lab itself with the materials used for this project had the most success with UnaG and IFP fluorescence relative to the others. This was reflected again in the final results of this project. The reason for results differing from literature values could be due to

the differences between the efficacies of the promoters used, protein stability, access to the chromophore (flavin) or the microbe being utilized (e.g., *B. thetaiotaomicron*).

Use of Antibiotic Pairings:

The construction of plasmids containing antibiotic resistance genes and fluorescent protein (FP) genes was used to allow future comparison between selection methods using only FPs or antibiotic resistance genes. The insertion of gene cassettes into the genomic DNA of cells needed to be confirmed with PCR to ensure that random mutations within the population of WT bacteria were not responsible for cell lines obtaining antibiotic resistance.

Fluorescence Testing of Potential Gene Markers:

Analysis of fluorescence data indicated that only one of the four original proteins was significantly fluorescing: UnaG. A fifth protein, IFP, was introduced to compensate, as two FPs were needed to continue. IFP (red) was chosen due to its high efficacy in previous works of the Degnan Lab. Bilirubin and biliverdin, ligands of UnaG and IFP respectively, can be easily added to the growth medium and do not affect growth of *B. thetaiotaomicron*. These molecules are also available in the mammalian gastrointestinal tract. Testing was performed with, and without cells, both groups had subgroups with and without bilirubin to ensure it was not presenting a confounding factor. The most efficient concentration of bilirubin to promote fluorescence of UnaG was found by the Degnan Lab to be 0.25 μM; however, in these experiments 2.4 μM of bilirubin consistently provided greater fluorescence. It is unclear what experimental variables are responsible for this difference.

Attachment Site and Its Effect on Fluorescence:

The attachment site of UnaG was cross-referenced with the fluorescence intensities measured to determine the effect the attachment location had on fluorescence (Figure 3). The

data was bimodal, with two intensity ranges containing most of the fluorescence intensities; however, the insertion location did not correlate with fluorescence. Fortunately, the two clusters of fluorescence values were close enough to make it clear that it would not affect the outcome of the rest of the procedure. Repeated testing showed that the two clusters of intensities remained consistent between test strains; however, no source was ever determined for this effect. For the purposes of continuing the experiment, it was deemed unnecessary to find the reason the data was bimodal.

tRNA Adjustment to UnaG:

It was known from previous research on the topic that the levels of different tRNA anticodons varied from species to species (Santos & Del-Bem, 2022). Some species would have minimal amounts of certain tRNA anticodons only for the basic purpose of surviving silent mutations, but these species still possessed primary tRNAs representing each amino acid they required. Having primary anticodons for each AA increased the efficiency of gene translation which was an effect that could theoretically be taken advantage of by those attempting to have a given species produce large amounts of a protein. The first gene sequence for UnaG used in this procedure was a tRNA-adapted form of UnaG from the Japanese Eel, *Anguilla japonica* (Kumagai et al., 2013). It was tRNA adapted again by the Degnan lab because it was believed it would lead to greater fluorescence. For this project, a previous tRNA adjustment (Chia et al. 2020) and one created by the Degnan Lab directly were used. The fluorescent protein denoted as UnaG was thus tRNA adjusted. After confirmation of status and fluorescence testing, the data strongly suggested that the tRNA adjustment of the UnaG-encoding sequence yielded roughly 1.7 times more fluorescence (Figure 2). It was determined that this procedure should be repeated

in the future to verify the results, but they were strong enough to proceed with the larger experiment using the Degnan-Lab-tRNA-adjusted form of UnaG-sequences.

Transposon Mutagenesis and The Associated Risks:

After the insertion of the UnaG_Erm^{R} cassette into the pSAM plasmid. Unlike the pNBU2 plasmid that introduced gene sequences to specific places in the target genome, this was far more random, potentially integrating into a majority of mobile genetic elements in the target genome. Unfortunately, this random insertion comes with some risks for the cell. Many insertion locations within the genome of the cell may have knocked out genes critical for survival, thus killing some cells. A mild version of this effect was noted as cells that were confirmed through antibiotic resistance screening to possess the inserted cassette had varying rates of growth under standard conditions: A possible reason for this observation is the loss of function of important (but non-critical) genes for growth. It is also worth noting that conjugation times were longer in this procedure than was suggested by Goodman et al. (2009) to result in primarily only one insertion in each donor.

Difficulties Preparing Recipient Strains:

As noted in the procedure section, the original intention was for *B. fragilis* to form the base for the recipient strains of the final cross of this experiment. pNBU2 proved rather ineffective at introducing the desired gene cassette into the genome of *B. fragilis*. Although the reason was never fully determined, it was noted that *B. fragilis* has a functioning CRISPR system, a bacterial defense against foreign DNA (Tajkarimi & Wexler, 2017). It was suspected that *B. fragilis's* CRISPR system was responsible for its unworkably low conjugation efficiency.

The subsequent substitution of *B. fragilis* for *B. ovatus*, yielded the successful production of a strain possessing resistance to tetracycline and the capacity to fluoresce red. Multiple colonies of *B. ovatus* (all shown to be potential recipients) were collected and stored.

Analysis of the Donor and Recipient Cross:

Neither of the two mating attempts resulted in colonies possessing the expected antibiotic resistance.

Unfortunately, it cannot be ruled out that transfer will occur in future repeats of this experiment. No stressors were used to stimulate transfer which theoretically would have increased the odds of horizontal gene transfer. This is because some MGEs such as dormant bacteriophages only become active when cells are stressed (Foster, 2007). Additionally, the second mating attempt involved the use of faulty materials, including antibiotic plates with subinhibitory levels of the critical antibiotic erythromycin. This resulted in some colony growth of donors when only successfully mated recipients should have survived. Future experiments with proper materials may yet allow for the creation and isolation of true, successfully mated recipients.

Future Directions:

Transposon mutagenesis can be a viable means to track activity of MGEs (J. Ortanez unpublished). However, so far the use of the UnaG Tn cassette has not yielded any confirmed transconjugants. I anticipate that the data collected here will inform future researchers in the Degnan Lab attempting to repeat this experimental procedure. It is possible that using different donors and/or recipient strains that are more compatible could lead to better results. Also, altering the growth conditions to induce stress or perhaps use an *in vivo* model may aid in observing transfer events of fluorescently labeled MGEs.

REFERENCES

BD Sciences. (2023). *What is multicolor flow cytometry: Cell sorting techniques.* What is Multicolor Flow Cytometry | Cell Sorting Techniques. Retrieved April 25, 2023, from https://www.bdbiosciences.com/en-us/learn/applications/multicolor-flow-cytometry#Overview

Bull, M. J., & Plummer, N. T. (2014). Part 1: The Human Gut Microbiome in Health and Disease. *Integrative medicine (Encinitas, Calif.)*, *13*(6), 17–22.Campbell, D. E., Ly, L. K., Ridlon, J. M., Hsiao, A., Whitaker, R. J., & Degnan, P. H. (2020). Infection with bacteroides phage BV01 alters the host transcriptome and bile acid metabolism in a common human gut microbe. *Cell Reports*, *32*(11), 108142. https://doi.org/10.1016/j.celrep.2020.108142

Chia, H. E., Zuo, T., Koropatkin, N. M., Marsh, E. N. G., & Biteen, J. S. (2020). Imaging living obligate anaerobic bacteria with bilin-binding fluorescent proteins. *Current Research in Microbial Sciences*, *1*, 1–6. https://doi.org/10.1016/j.crmicr.2020.04.001

Degnan, P. H., Barry, N. A., Mok, K. C., Taga, M. E., & Goodman, A. L. (2014). Human gut microbes use multiple transporters to distinguish vitamin B_{12} analogs and compete in the gut. *Cell host & microbe, 15*(1), 47–57. https://doi.org/10.1016/j.chom.2013.12.007

Foster, P. L. (2007). Stress-induced mutagenesis in bacteria. *Critical Reviews in Biochemistry and Molecular Biology*, *42*(5), 373–397. https://doi.org/10.1080/10409230701648494

Frye, K. A., Piamthai, V., Hsiao, A., & Degnan, P. H. (2021). Mobilization of vitamin B12 transporters alters competitive dynamics in a human gut microbe. *Cell reports, 37*(13), 110164. https://doi.org/10.1016/j.celrep.2021.110164

Goodman, A. L., McNulty, N. P., Zhao, Y., Leip, D., Mitra, R. D., Lozupone, C. A., Knight, R., & Gordon, J. I. (2009). Identifying genetic determinants needed to establish a human gut symbiont in its Habitat. *Cell Host & Microbe*, *6*(3), 279–289. https://doi.org/10.1016/j.chom.2009.08.003

Kumagai, A., Ando, R., Miyatake, H., Greimel, P., Kobayashi, T., Hirabayashi, Y., Shimogori, T., & Miyawaki, A. (2013). A bilirubin-inducible fluorescent protein from EEL MUSCLE. *Cell, 153*(7), 1602–1611. https://doi.org/10.1016/j.cell.2013.05.038

Lobo, L. A., Smith, C. J., & Rocha, E. R. (2011). Flavin mononucleotide (fmn)-based fluorescent protein (FbFP) as selectable marker for gene expression in the anaerobe bacteroides fragilis. *FEMS Microbiology Letters, 317*(1), 67–74. https://doi.org/10.1111/j.1574-6968.2011.02212.x

Martens, E.C., Chiang, H.C. and Gordon, J.I. (2008) 'Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont', *Cell Host & Microbe, 4*(5), pp. 447–457. doi:10.1016/j.chom.2008.09.007.

Mukherjee, A. et al. (2014) 'Engineering and characterization of new LOV-based fluorescent proteins from chlamydomonas reinhardtii and vaucheria frigida', *ACS Synthetic Biology, 4*(4), pp. 371–377. doi:10.1021/sb500237x.

Santos, F.B. and Del-Bem, L.-E. (2022) 'The evolution of trna copy number and repertoire in Cellular Life', *Genes, 14*(1), p. 27. doi:10.3390/genes14010027.

Tajkarimi, M., & Wexler, H. M. (2017). CRISPR-cas systems in bacteroides fragilis, an important pathobiont in the human gut microbiome. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.02234

Wang, J., Shoemaker, N. B., Wang, G.-R., & Salyers, A. A. (2000). Characterization of a bacteroides mobilizable transposon, NBU2, which carries a functional lincomycin resistance gene. *Journal of Bacteriology*, *182*(12), 3559–3571. https://doi.org/10.1128/jb.182.12.3559-3571.2000